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(54) Title: METHOD OF PURIFYING CROSS-LINKED HEMOGLOBIN

(57) Abstract

A method of purifying cross-linked hemoglobin which is mixed with non-cross-linked hemoglobin, which comprises heating the hemoglobin mixture at a temperature of about 60 degrees to 85 degrees C for a time sufficient to cause the precipitation of a substantial amount of the non-cross-linked hemoglobin present. Thereafter, one separates the precipitate thus formed from the cross-linked hemoglobin.

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METHOD OF PURIFYING CROSS-LINKED HEMOGLOBIN

TECHNICAL FIELD

A considerable amount of research has been directed to the development of a substitute for red blood cells as an oxygen carrier in the circulatory system of a living patient. This research has been prompted by the fact that these substitutes offer several potential advantages over the use of whole blood or red blood cells. As one advantage, an artificial oxygen carrier would not require typing and cross-matching as is the case for red blood cells. As another advantage, an artificial oxygen carrier would be very likely free of the risk of AIDS or hepatitis transmission. A third advantage of red cell substitutes is the potential for long term storage.

Early experiments in this field evaluated the use of stroma-free hemoglobin as a red cell substitute. When stroma-free hemoglobin is infused intravenously into animals, its serves briefly as an oxygen carrier in the circulatory system. Unfortunately, however, the natural hemoglobin tetramer dissociates into lower molecular weight components which are readily excreted through the kidney. Accordingly, free hemoglobin is rapidly removed by the body from circulation, which seriously limits the usefulness of free hemoglobin as an oxygen carrying blood substitute.

In an attempt to prevent the fast excretion of hemoglobin from the body, a number of researchers have evaluated the covalent cross-linking of hemoglobin, both intramolecular cross-linking in which the molecular weight of the product is not increased to a great degree, and intermolecular cross-linking, in which the molecular weight of the product is

significantly greater than the molecular weight of natural hemoglobin (about 64,000). Typical cross-linking agents used are glutaraldehyde and 3,5-dibromosalicyl-bis-fumarate. See for example the following U.S. Patents which deal with such subjects: Mazur U.S. Patent No. 3,925,344; Bonhard et al. U.S. Pat. No. 4,336,248; Bonsen U.S. Pat. Nos. 4,001,200; 4,001,401; and 4,053,590; Morris et al. U.S. Pat. No. 4,061,736; Tye U.S. Patent No. 4,529,719; Walder U.S. Pat. Nos. 4,598,064 and 4,600,531; and Netherlands Patent No. 7404140. A considerable amount of technical literature also exists on the subject.

When hemoglobin is reacted with cross-linking agents, the product is almost always a mixture of products including substantial amounts of unmodified, non-cross-linked hemoglobin. While some of the cross-linked hemoglobins exhibit significant promise as a cell-free blood substitute, it is deemed desirable to remove the unmodified hemoglobin since upon infusion it will be rapidly removed by the kidneys anyway, and there is some evidence that large amounts of free, non-cross-linked hemoglobin may exhibit toxicity. In the known prior art, unmodified, non-cross-linked hemoglobin is removed from cross-linked hemoglobin by techniques such as chromatography. Chromatography is a laborious and expensive method which imposes severe constraints on the amount of purified, cross-linked hemoglobin which can be produced.

Additionally, in some cases the separation of non-cross-linked from cross-linked hemoglobin is technically difficult, as well as expensive, because byproducts of the reaction become irreversably bound to the chromatographic column, which r duces the functional capacity of the column.

In accordance with this invention, a method for purification of cross-linked hemoglobin is provided which is greatly simplified in comparison with chromatography, and which is of substantially reduced cost as well. Thus the manufacture of cross-linked hemoglobin is greatly facilitated.

DESCRIPTION OF THE INVENTION

In this invention, a method is provided for purifying cross-linked hemoglobin which is mixed with non-cross-linked hemoglobin. In accordance with this invention, the cross-linked hemoglobin is heated at a temperature of about 60 degrees to 85 degrees C. for a time sufficient to cause the precipitation of a substantial amount of the non-cross-linked hemoglobin (and frequently other impurities as well) present, without precipitating major amounts of cross-linked hemoglobin. Thereafter, one separates the precipitate formed from the cross-linked hemoglobin, typically by centrifugation or filtration.

Preferably, the cross-linked hemoglobin is processed in accordance with this invention at a pH of 6.5 to 9, most preferably a pH of 7 to 8, and specifically a pH of about 7.5. The duration of the heating step is preferably substantially one to six hours.

In one embodiment of this invention, the hemoglobin may be maintained in the substantially deoxygenated state during heating. This can be accomplished by various solution degassing procedures. These include, but are not limited to, sparging with inert gases; circulation through membrane gas exchange devices; and exposing hemoglobin solutions to a

vacuum. The suitability of such procedures will be limited by the extent that they promote degradation of hemoglobin, for example through foaming, acidification, or the like. For example, one may pass hemoglobin through a membrane oxygenator, for example a model number 08-2A membrane oxygenator of Sci-Med Life Systems Inc. of Minneapolis, Minnesota, with the gas channel of the oxygenator being filled with flowing nitrogen or argon. By such procedure, the hemoglobin may be deoxygenated, followed by heating in accordance with this invention in a sealed, oxygen-free container to prevent reoxygenation. As another alternative, hemoglobin solutions may be sparged with an oxygen-free inert gas such as nitrogen or argon making use, for example, of a well known bubble-type oxygenator.

Alternatively, the hemoglobin may also be maintained in its deoxy form using an appropriate reducing agent. Such a reducing agent generally is a chemireductant which should be physiologically acceptable and will typically have a reducing potential greater than or more effective than ascorbate against hemoglobin. Reduced redox dyes and sulfhydryl or sulfoxy compounds include many acceptable reducing agents. Suitable reducing agents also may include alkali metal (e.g. sodium or potassium) dithionites, bisulfites, metabisulfites, or sulfites. Other soluble, non-toxic salts of such anions may be candidates for use as well. Additionally, reduced glutathione or dithiothreitol may be used as well.

The quantity of reducing agent to be included in the hemoglobin composition (The hemoglobin is typically present in buffered aqueous solution in a concentration of preferably 1 to 10 grams per

deciliter) may vary, depending upon the reducing strength of the agent, the quantity of hemoglobin present, the temperature and duration of heating exposure, the presence of oxidizing agents, and other factors as will be apparent to the skilled artisan. Accordingly, the optimal concentration will be determined by routine experiments, for example by following the changes in hemoglobin composition as determined by ion exchange high performance liquid chromatography during the heating process. Dithionite may typically be used in a concentration of about 10 to 100 mM in hemoglobin solutions, preferably about from 20 to 40 mM (expressed in terms of mM per litre).

Other preferred reducing agents which may be used include glutathione, N-acetyl-L-cysteine, and N-2-mercapto-propionyl glycine.

It is generally preferred to heat the deoxygenated solution containing both cross-linked and non-cross-linked hemoglobin and its impurities at a concentration of 1-10 grams per deciliter and a solution pH of 7 to 8. The heating may be at about 65 or 70 to 80 degrees C. for about 1-6 hours under an inert atmosphere of typically nitrogen or argon. In such a process, a precipitation of non-cross-linked hemoglobin will take place, along with other by-products of the reaction in which the cross-linked hemoglobin was formed. Following the heating step, the resulting precipitate can be removed by centrifugation and/or filtration, while a large percentage of the cross-linked hemoglobin does not precipitate and remains in solution.

Alternatively, the hemoglobin may be maintained in substantially the oxygenated state during heating. Under this circumstance, a buffered solution of hemoglobin at concentration and pH

preferably in the ranges described above may be heated preferably from about 60 to 75 degrees C., typically at about 65 degrees C., for a period of time of about 1 to 6 hours, for example one and one-half hours. The non-cross-linked hemoglobin precipitates from solution while the cross-linked hemoglobin remains substantially in dissolved form. Then, filtration and/or centrifugation may take place to remove the precipitate, composed primarily of non-cross-linked hemoglobin and other protein impurities.

However, in this circumstance, it is likely that substantial conversion of the cross-linked hemoglobin to methemoglobin will take place. Accordingly, in this circumstance a subsequent step will take place in which methemoglobin present is reduced by reaction with a reducing agent of a type described above, for example an alkali metal dithionite in the proportions stated above, to regenerate functional cross-linked hemoglobin once again.

Typically, reagents which may be used to crosslink non-cross-linked hemoglobin may be glutaraldehyde, dextran, polyethylene glycol, and the like, with specific processes for producing cross-linked hemoglobin being as specifically described in the patents cited above. Specifically, the cross-linked hemoglobin may be prepared as described in Walder U.S. Patent No. 4,600,531.

Example 1

A crude reaction mixture of diaspirin cross-linked hemoglobin was prepared by adding 1.5 equivalents of dibromosalicyl-bis-fumarate (DBBF) to a

deoxygenated solution containing 3 g/dL stroma-free hemoglobin, 10 mM sodium phosphate buffer, pH 7.0, and 10 equivalents of inositol hexaphosphate (IHP). The solution was stirred at 37 degrees C. for 2 hours. Several aliquots of this solution were removed, the pH adjusted to 7.4, and the aliquots deoxygenated by repeated, alternating exposure to vacuum and nitrogen by flushing and evacuating the hemoglobin in a small vessel six or seven times. The aliquots were then heated at 70 degrees C. for varying lengths of time, and the precipitate formed was removed by centrifugation. The supernatants were analyzed for hemoglobin content and composition. Total hemoglobin and percent methemoglobin were determined spectrophotometrically, while the amount of cross-linked hemoglobin present was assessed by ion-exchange high performance liquid chromatography (HPLC). The latter procedure can distinguish residual unmodified hemoglobin from the desired intramolecularly cross-linked product.

The results of this study (Table 1) demonstrate that under these experimental conditions the unmodified hemoglobin is selectively precipitated from solution. At higher temperatures we found that both types of hemoglobin were precipitated from solution. These results demonstrate that under the appropriate conditions it is possible to selectively precipitate unmodified hemoglobin from a crude reaction mixture containing a cross-linked derivative.

Table 1

Composition of a Reaction Mixture of Diaspirin (DBBF) Cross-linked Hemoglobin During Heating at a pH of 7.4 at 70 degrees C

Time of Heating (hr.)	% of Original Protein Component Remaining	
	Unmodified Hemoglobin	Diaspirin Cross-linked Hemoglobin
0	100	100
1	62	95
2	17	94
3	15	94

Example 2

A crude reaction mixture of diaspirin cross-linked hemoglobin was prepared as described in Example 1 and the solution rendered free of ions such as IHP, glycine, and 3,5-dibromosalicylate by diafiltration and chromatography on a Sephadex G-25 column. The hemoglobin-containing eluate was adjusted to a pH of 7.4, deoxygenated, and aliquots heated at 80 degrees C. for up to two hours. Samples were analyzed for hemoglobin content as described in Example 1. The results of this experiment (Table 2) demonstrate that the removal of one or more of the small molecules present in crude reaction mixtures results in enhanced thermal stability of h moglobins in general, but that

selective precipitation of the unmodified molecules is still possible by increasing the temperature to 80 degrees C.

Table 2

Composition of a Diafiltered and Chromatographically Purified Reaction Mixture During Heating at a pH of 7.4 at 80 C

Time of Heating (hr.)	% of Original Protein Component Remaining	
	Unmodified Hemoglobin	Cross-linked Hemoglobin
0	100	100
1	34	85
2	15	84

Example 3.

Hemoglobin was prepared from outdated blood by hypotonic lysis with distilled water. Stroma was removed by centrifugation of the suspension at 35000 x g for one hour. The cross-linking reaction was performed in bis-tris buffer, pH 7.2, under anaerobic conditions established by purging with nitrogen. The solution contained 1 mM hemoglobin and 5mM inositol hexaphosphate. After adding 1.5 equivalents of DBBF the reaction was allowed to proceed for 2 hours at 37

degrees C and then stopped by the addition of an equal volume of 2 M glycine adjusted to a pH of 8.0 with NaOH. The ratio of cross-linked product to unmodified hemoglobin as determined by analytical isoelectic focusing was 4:1.

After the cross-linking reaction the sample was oxygenated with room air and then heated to 65 degrees C for 1.5 hours. This lead to precipitation of 32% of the total hemoglobin present including all of the unmodified hemoglobin. The hemoglobin remaining in the supernatant contained 66% methemoglobin. The precipitated hemoglobin was removed by centrifugation and filtration through a sterile 0.22 micron pore-size membrane. The sample was then cooled to 4 degrees C, deoxygenated, and sodium dithionite added to a final concentration of 40 mM in order to reduce the methemoglobin back to the unoxidized form. The reaction was allowed to proceed for five minutes, and the excess dithionite was subsequently removed by gel filtration over a Sephadex G-25 column maintained under anaerobic conditions by purging the buffer with nitrogen. The final product contained 5% of the hemoglobin in the met form.

This experiment demonstrates that heat treatment under aerobic conditions may be used to selectively denature and precipitate residual unmodified hemoglobin in reaction mixtures, and that the purified methemoglobin form of the crosslinked product resulting from this treatment may be subsequently chemically reduced back to the unoxidized form.

The above has been offered for illustrative purposes only, and is not intended to limit th scope of the invention, which is as defined in the claims below.

THAT WHICH IS CLAIMED IS:

1. The method of purifying cross-linked hemoglobin which is mixed with non-cross-linked hemoglobin, which comprises:

heating said hemoglobin mixture at a temperature of 60 degrees to 85 degrees C. for a time sufficient to cause the precipitation of a substantial amount of the non-cross-linked hemoglobin present, and thereafter separating the precipitate thus formed from the cross-linked hemoglobin.

2. The method of Claim 1 which is performed at a pH of 6.5 to 9.

3. The method of Claim 2 which is performed at a pH of essentially 7 to 8.

4. The method of Claim 1 in which said heating has a duration of substantially 1 to 6 hours.

5. The method of Claim 1 in which said hemoglobin mixture is maintained in substantially the deoxygenated state during heating at a temperature of at least 65 degrees C..

6. The method of Claim 1 in which said hemoglobin mixture is maintained in substantially the oxygenated state during heating.

7. The method of Claim 6 in which, after said heating, methemoglobin present is reduced by reaction with a reducing agent to regenerate functional hemoglobin.

8. The method of Claim 1 in which said hemoglobin mixture is heated in aqueous solution at a concentration of 1-10 g. per deciliter of solution.

9. The method of Claim 8 in which said solution is maintained at a pH of essentially 7 to 8.

10. The method of purifying cross-linked hemoglobin which is mixed with non-cross-linked

hemoglobin, which comprises:

heating said hemoglobin mixture at a temperature of 65 degrees to 85 degrees C for a duration of substantially 1 to 6 hours at a pH of essentially 7 to 8 to cause the precipitation of a substantial amount of the non-cross-linked hemoglobin present, and thereafter separating the precipitate thus formed from the cross-linked hemoglobin.

11. The method of Claim 10 in which said hemoglobin mixture is maintained in the deoxygenated state during heating.

12. The method of Claim 11 in which said hemoglobin mixture is maintained in the deoxygenated state by the presence of a reducing agent.

13. The method of Claim 11 in which said hemoglobin mixture is maintained in the deoxygenated state by initial oxygen exchange from the hemoglobin into an inert, oxygen-free gas or vacuum, followed by keeping the hemoglobin under oxygen-free conditions.

14. The method of Claim 10 in which said hemoglobin mixture is heated in aqueous solution at a concentration of 1-10 grams per deciliter of solution.

15. The method of Claim 14 in which said solution is a pH of essentially 7 to 8.

16. The method of Claim 10 in which said hemoglobin mixture is maintained in substantially the oxygenated state during heating.

17. The method of Claim 16 in which, after said heating, methemoglobin present is reduced by reaction with a reducing agent to regenerate functional hemoglobin.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/01489

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): A61K 37/14

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	530/385; 514/6; 424/101

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

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III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0179075, ESTEP, Published 30 April 1986	1-17

* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
08 June 1989	25 JUL 1989

International Searching Authority	Signature of Authorized Officer
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